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Biochimica et Biophysica Acta 1740 (2005) 147–154
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Mechanisms of genomic and non-genomic actions of carotenoids

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Received 23 September 2004; received in revised form 19 November 2004; accepted 8 December 2004
Available online 30 December 2004

Abstract

Carotenoids are highly bioactive dietary compounds that have the potential to have significant effects on human health. It is becoming increasingly clear that the various biological effects that carotenoids exert could be driven via a number of different mechanisms. These include direct pro- and antioxidant effects, redox sensitive cell signalling, vitamin A signalling pathways and other as yet unidentified mechanisms. This article provides an overview of the known effects of carotenoids and discusses the use of model systems and functional genomic approaches further to elucidate their modes of action.

Keywords: Carotenoid; β-Carotene; Vitamin A; Gene expression; Functional genomics; Model system

1. Introduction

Carotenoids, a family of pigmented compounds found in the flesh of fruits and vegetables, are excellent candidates for compounds that contribute to the health promoting effects of these foods [1–3]. Much of the research to date has focused on the potential role of the carotenoids as dietary antioxidants [4,5]. There is little doubt that, under the right conditions, carotenoids can provide cells, tissues and other structures such as lipoproteins with a degree of antioxidant protection [6–9]. However, the true significance of these effects both under normal physiological conditions and conditions of oxidative stress remains unclear.

Some might argue that the initial focus of carotenoid research on their role as antioxidants has set back progress in research into other biological activities of these compounds. While it remains entirely possible that some of their biological effects result from changes in cellular redox status through redox-sensitive cell-signalling pathways [10], other mechanisms of action that are independent of their antioxidant properties are also likely to be important. One obvious example is that certain members of the family (such as α- and β-carotene and β-cryptoxanthin) can be processed within the body to yield vitamin A, a potent bioactive agent exerting profound effects on processes such as development and disease prevention. But by no means all the observed effects of carotenoids can be explained on this basis alone. Certain effects have been observed with pro-vitamin A carotenoids that are not elicited by vitamin A itself [11,12]. Equally, the non-provitamin A carotenoids also are capable of altering patterns of gene expression and cell function, sometimes similarly and sometimes quite differently to vitamin A [13].

While much experimental evidence has been accumulated demonstrating the potency and nature of the biological effects of carotenoids, in most cases their underlying mechanisms of action remain uncertain. This is due to the breadth of the range of biological effects observed and their tissue specificity, time- and dose-dependency, and limitations of the available model and delivery systems.

An opportunity to make much faster progress in unravelling this complex enigma has arisen with the development of functional genomic techniques. These provide the researcher with the tools necessary to investigate many or even all potential regulatory mechanisms and processes in

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doi:10.1016/j.bbadis.2004.12.009

parallel, to prepare comprehensive inventories of responses, to categorise the different carotenoids according to the pattern of effects they trigger and to define the signalling pathways involved.

This new opportunity is particularly timely in the light of the backlash suffered by the most prominent member of the carotenoid family, β-carotene. This has followed the overall lack of clear health benefits reported in a number of large prospective dietary intervention studies with detrimental effects reported in some [14–16]. Does this genuinely indicate that carotenoid research been misguided all along and that these compounds do not act in vivo to promote health other than providing a source of vitamin A? Alternatively, have the prospective studies failed to detect benefits owing to factors of study design, and lack of sufficiently sensitive biomarkers? For example, detrimental effects were observed most notably in studies using high chronic doses of β-carotene in "at risk" populations. A potential mechanism has been proposed that could explain the adverse effects of chronic pharmacological doses of β-carotene under conditions of oxidative stress, such as smoking [17–19]. These adverse effects are not likely to be observed at more realistic dietary intakes or in the absence of conditions of oxidative stress. Consequently, the poor public reputation that β-carotene has attained as a result of these studies is not truly justified, since it is based primarily on the concern that it could be dangerous rather than on the apparent lack of health benefits.

On the question of continuing β-carotene research, the lack of observed health benefits is the major issue. While the results of these studies should be the basis for careful reflection and consideration, they do not provide sufficient evidence to justify stopping β-carotene research entirely. The inevitable practical limitations of such large studies together with the restricted range of biomarkers and endpoints available make it impossible to be confident that all possible important biological effects have been detected.

Careful study design and development of new methodologies will be critical factors in making meaningful progress [20]. In studies involving human volunteers, the key tissues of interest often are not obtainable in sufficient quantity at serial sampling points or simply not available at all. This makes the use of model systems necessary. Indeed, these may be preferable in some cases, as it is far easier to maintain tight experimental control using models, minimising genetic and environmental variation, thus increasing the likelihood of successfully detecting relatively subtle effects.

A very careful consideration of the model systems to be used is essential for carotenoid research (discussed below). Most animals do not absorb and metabolise carotenoids in the same way as humans do. The suitability of an animal model varies, to some extent, depending on the particular carotenoid under investigation [21].

Another approach is to use cell culture systems. These can be regulated even more closely than animals and present the clear advantage of the simplicity of using a single cell type rather than the multiple cell types present in most tissue samples. However, most cell culture systems use immortalised or cancer cells that can no longer be considered truly equivalent to normal cells. Even primary cells in culture may not behave exactly as they would in vivo because of their separation from other cell types and normal body signalling mechanisms (such as hormones) and the highly non-physiological environment of the incubator. Furthermore efficient and representative delivery of the lipophilic carotenoids to cells in culture presents a particular technical challenge.

This paper seeks (i) to summarise the evidence of different biological effects and proposed mechanisms for carotenoid bioactivity, (ii) to address some of the advantages and disadvantages of different model systems and (iii) to review the data obtained with functional genomic techniques for carotenoids and related compounds to date.

2. Anti- and pro-oxidant effects of carotenoids

Under some circumstances carotenoids may act as cellular antioxidants. For example, β-carotene can suppress the up-regulation of haem oxygenase-1 gene expression provoked by UVA exposure in human dermal fibroblasts (FEK4) in a dose-dependent manner [22]. This is consistent with a direct antioxidant (singlet oxygen quenching) effect as is the observation that UVA exposure caused the depletion of cellular β-carotene and the accumulation of apo-carotenal. However, the activation of retinoid signalling via retinoic acid receptors RARs and RXRs could not be ruled out as a possible alternative mechanism. Curiously, the authors of this study also reported that at the lowest level used (0.2 μM) the presence of β-carotene actually augmented UVA induced haem oxygenase-1 induction. Furthermore, others have reported that β-carotene can act as a pro-oxidant augmenting the induction of haem oxygenase-1 in human skin fibroblasts (HFP-1) [23]. One key difference between these two studies was the delivery vehicle used for the β-carotene. As discussed below, this kind of technical issue could have a profound effect on the outcome of such studies.

In another study β-carotene, at high concentration (>10 μM), appeared to be able to act as a pro-oxidant in vitro increasing the production of reactive oxygen species and cellular oxidised glutathione content [24]. This was associated with the activation of NF-kB, the induction of c-myc expression, the promotion of apoptosis and the inhibition of cell growth in human leukaemia and colon adenocarcinoma cell lines. In the same system, α-tocopherol and *N*-acetylcysteine blocked these effects of β-carotene.

Rats supplemented with β-carotene exhibit increases in phase I enzymes in lung, liver, kidney and intestine together with the production of oxidative stress [25,26]. Here again, α-tocopherol and *N*-acetylcysteine reduce the

effects of β-carotene, presumably by reducing the over-production of reactive oxygen species or up-regulation of phase I enzymes, respectively [27].

Oxidative stress induced in these conditions by β-carotene will promote the production of oxidative break-down products of the β-carotene, such as β-apo-carotenals. These oxidised products, which are found particularly in smokers, may further promote carcinogenesis [28]. A study in which ferrets were supplemented with high dose β-carotene and exposed to cigarette smoke suggests that the underlying mechanism involves the suppression of retinoid signalling, due to reduced RAPβ gene expression, and the activation of AP-1 [18].

However, in the ATBC study, there was no evidence of an interaction between α-tocopherol and β-carotene with respect to the incidence of lung cancer in the human volunteers [16]. Thus if additional oxidative stress was the primary cause of the increased lung cancer incidence in the volunteers receiving β-carotene supplementation, co-supplementation with α-tocopherol was not sufficient to correct for this.

3. Effects of carotenoids on immune function

Dietary carotenoids modulate immune function in a number of animal models. These include stimulation of blood neutrophil killing activity via increased myeloperoxidase and phagocytic activity, increased mitogen-induced lymphocyte proliferation, enhanced antibody response, and increased cytochrome oxidase and peroxidase activities in macrophages, indicating increased respiratory burst [13].

These effects are not due solely to the actions of vitamin A since they have been observed both with pro-vitamin A carotenoids in animal models that are highly inefficient at converting them to vitamin A and with non-pro-vitamin A carotenoids [13]. For example, lycopene increases T helper cells and normalises intra-thymic T cell differentiation resulting from tumourigenesis in mice [29]. β-Carotene stimulates cattle blood neutrophil bacterial killing activity via increased myeloperoxidase activity and phagocytic activity while vitamin A generally decreases phagocytosis and does not alter killing activity [11,12].

4. Anticarcinogenic effects

In addition to the epidemiological evidence, carotenoids (and retinoids) have been demonstrated to exert chemoprotective effects in a range of animal tumour models and cell lines. A number of possible mechanisms of action have been proposed but which, if any, of these are most physiologically significant is still unclear. The following sections provide a brief overview of some of the evidence and proposed mechanisms of action.

4.1. Effects on tumour cells in culture

β-carotene inhibits the growth of MCF-7 and Hs578T cancer cells. Lycopene, but not canthaxanthin, inhibits the growth of MCF-7 and MDA-MB-231 human breast cancer cells [30]. Both retinoic acid and palm oil carotene concentrate (containing primarily α- and β-carotene with some lycopene and phytoene) provoke a dose-dependent inhibition of cell growth in oestrogen receptor-positive breast cancer cells (MCF-7) but not in oestrogen receptor-negative cells (MDA-MB-231). The palm oil carotene concentrate did not alter the expression of the oestrogen-regulated pS2 gene in MCF-7 cells, which is not expressed at all in MDA-MB-231 cells, whereas retinoic acid did suppress its expression [31]. These data suggest that the presence of oestrogen receptor is important though not essential for these actions of carotenoids on mammary tumour cell growth.

β-Carotene has been shown to inhibit the growth of five human colon adenocarcinoma and three prostate cell lines in vitro independently of p53 and/or p21WAF1 gene expression [32,33]. However, since retinol was detected in the prostate cell lines following incubation with β-carotene, there is the possibility that this may be the active agent responsible for the growth inhibition.

α-Carotene arrests GOTO neuroblastoma cells in the GO–G1 phase of cell cycling and does so ten times more potently than β-carotene [34]. This is associated with the suppression of N-myc transcript levels.

Lycopene and α- and β-carotene inhibit the growth of human endometrial, mammary and lung cancer cells lines [35]. Lycopene also acts synergistically with vitamin D3 to inhibit cell cycle progression and induce differentiation in HL60 promyelocytic leukaemia cell line [36].

4.2. Effects on tumour growth and immunity

Astaxanthin, canthaxanthin, β-carotene and lutein inhibit the growth of transplantable mammary tumours in mice [37–39]. The presence of the tumour in this model was associated with reduced populations of T, Th, and Tc cells but increased populations of IL-2Rα and B cells [40]. Lutein prevented these changes and also increased IFN-γ gene expression and decreased the expression of IL-10 in splenocytes of tumour-bearing mice. IFN-γ is a potent inducer of macrophage activation and class II molecules. IL-10 inhibits IFN-γ production, antigen presentation, IL-1, IL-6 and TNFα production by macrophages. Additionally, lutein decreased apoptosis in blood leukocytes from tumour-bearing mice but increased apoptosis of tumour cells [41].

Dietary lycopene delays the onset and reduces the growth of mammary tumours in a mouse strain with a high spontaneous incidence [42]. In this case, dietary lycopene was associated with a reduction in the activity of thymidylate synthetase in the mammary tissue and reduced serum free fatty acid and prolactin concentrations. Lycopene also

exerts chemopreventive effects in chemically-induced mouse lung neoplasia [43].

Both β-carotene and retinoic acid reduce the incidence and growth of chemically-induced liver tumours in rat and also modulate cellular antioxidant defence and xenobiotic detoxification processes in this model [44]. In human volunteers dietary supplementation with β-carotene was found to increase the percentage of blood monocytes expressing the major histocompatibility complex class II molecule HLA-DR, the adhesion molecules intercellular adhesion molecule-1 and leukocyte function-associated antigen-3 and the secretion by monocytes of TNF-α [45].

4.3. Effects on apoptosis and necrosis

β-Carotene treatment decreases levels of the anti-apoptotic proteins Bcl-2 and Bcl-xl but does not alter the expression of pro-apoptotic BAX in human colon adenocarcinoma cell lines [33]. Lutein induces the expression of p53 and BAX, suppresses the expression of Bcl-2 and decreases angiogenic activity in transplantable mouse mammary tumours [41]. Similarly, lutein also selectively induces apoptosis in transformed cells in vitro, while protecting normal cells from apoptosis induced by chemotherapy agents by selectively increasing the Bcl-xL:Bax protein ratio [46].

Pre-supplementation of rats with lycopene was associated with an increased necrotic area of prostate tumours produced by the injection of MatLyLu prostate tumour cells [47]. It is suggested that this effect is mediated through reduced expression of genes involved with steroid metabolism and signalling.

5. Other biological effects of carotenoids

Carotenoids may also influence cell function through changes in membrane fluidity and cell–cell communication via gap junctions [48,49]. The modulation of gap junction communication (via regulation of connexin 43 expression) correlates closely with the ability of canthaxanthin, β-carotene and lycopene to inhibit the transformation by 3-methylcholanthrene of C3H10T1/2 cells in vitro [49]. There is also evidence that this mechanism could play a role in cancer protection in vivo as the results from a small phase II study have suggested that dietary lycopene can reduce tumour growth and levels of prostate-specific antigen and increase the expression of connexin 43 in tumour tissue from human prostate cancer patients [50]. This regulation of gap junction communication is not due exclusively to provitamin A activity and does not appear to be due to antioxidant activity since other antioxidants such as α-tocopherol do not exert the same effect.

In relation to cardiovascular disease, in vitro and in vivo evidence suggests that lycopene and β-carotene can act as hypocholesterolaemic agents by inhibiting the HMG-CoA reductase pathway and augmenting the expression of the LDL receptor [51,52]. β-carotene, lutein and lycopene all alter the expression of cell surface adhesion molecules on human enthothelial cells in vitro in a manner that would be expected to reduce the binding of monocytes [53].

It has also been suggested that carotenoids could influence mitochondrial function via at least two possible mechanisms. Firstly, the function of F_1F_0ATPase can be modulated by mitochondrial membrane fluidity affecting the motion of the F0 component [54]. Dietary fats and lipophilic compounds such as carotenoids may, therefore, alter mitochondrial function through this mechanism. Secondly, there is a putative retinoic acid response element in the D loop of the mitochondrial genome. Retinoic acid receptors have been found in the mitochondrial compartment and it has been demonstrated that increasing the vitamin A content of the diet in rats increased oxidative phosphorylation activity, ATPase 6 gene expression and some other mitochondrial genes [54].

6. Use of model systems

6.1. Animal models

No single animal model accurately mimics all aspects of human absorption and metabolism of carotenoids. Humans absorb a variety of carotenoids intact with a variable proportion of the pro-vitamin A carotenoids cleaved, either in the intestinal epithelium or elsewhere in the body, to yield retinol. When designing an experiment the most appropriate model should be selected on the basis of its ability to absorb and metabolise the specific carotenoid or carotenoids of interest in a manner representative of humans and for its validity as a model of a specific disease endpoint if this is to be considered too. For example, gerbils, ferrets and pre-ruminant calves may, in general, be considered the best available models for carotenoid uptake and metabolism. However, care must still be taken in selecting these animals as they are not all entirely appropriate for the study of all relevant diseases states such as cancer, cardiovascular disease, immune function, etc. Both the practical and theoretical advantages and disadvantages of the different animal models have been reviewed comprehensively elsewhere [21,55].

Transgenic and gene knock-out technologies now make possible the generation of animal models tailored for the investigation of the regulation of specific processes in vivo including those influenced by nutrients and micronutrients. An excellent example of the potential use of these technologies is the transgenic mouse model that expresses the luciferase gene under the control of the NF-kB promoter. This has already been used to demonstrate how vitamin A status regulates NF-kB activity [56].

6.2. Cell culture systems: uptake and sub-cellular localisation of carotenoids

Delivery of carotenoids to cultured cells is challenging due to their hydrophobic properties. Additionally there are concerns about the stability of carotenoids added to the culture medium [57,58]. It is quite possible that some of the biological effects that have been observed using in vitro models are due to the effects of breakdown products rather than the intact carotenoid.

In vivo, carotenoids are delivered to most cells via lipoproteins and so these should be considered the ideal model for in vitro delivery of carotenoids as well. The cellular uptake observed with carotenoid-enriched lipoproteins appears to be at least as good as that achieved with most synthetic delivery vehicles [57,58]. Furthermore, β-carotene stability is markedly enhanced when it is added to cell culture medium as an integral component of lipoproteins compared to other delivery vehicles. However, the production of lipoprotein preparations that are sufficiently enriched in the desired carotenoid is not straightforward. If human volunteers are used as donors, a degree of enrichment may be achieved by prior dietary supplementation but the extent will vary from person to person and from carotenoid to carotenoid. Additionally, all such lipoprotein fractions unavoidably will contain differing quantities of a range of other carotenoids and bioactive components that are derived from the normal diet.

Somewhat greater control might be achieved using lipoproteins derived from animals following dietary supplementation. However, this is still a very labour intensive approach, the degree of enrichment that may be achieved is limited and the validity of the delivery systems is somewhat compromised by the species difference that is introduced.

An interesting alternative might be to use cultured human intestinal epithelial cells as factories for producing carotenoid-enriched chylomicrons. Once confluent and differentiated, the human Caco-2 epithelial cell model has been demonstrated to be capable of secreting chylomicrons when supplemented with oleic acid and taurocholate [59]. When micellar β-carotene was also added to this system it was found to be taken up into the cells relatively efficiently (up to 11% of total) and transferred into secreted chylomicrons (80% of total absorbed) [60]. However, the scope for producing sufficient quantities of carotenoid-enriched chylomicrons in this way, the stability of the carotenoid in these lipoprotein particles and their interaction with other cultured cells have not been evaluated properly. Consequently, the majority of the available research literature describes studies in which more straightforward delivery vehicles are used. These include water-soluble organic solvents, water-dispersible beadlets, micelle and liposome preparations and cyclodextrins.

Carotenoid stability and cellular uptake vary significantly with these different delivery vehicles [22,32,61]. Furthermore, the extent of cellular uptake varies from cell type to cell type, from carotenoid to carotenoid and between different isomeric forms [6,32,53,60]. Some of this variation may correspond to genuine biological differences. Other differences may be artefacts due to the non-physiological mode of delivery or the delivery vehicle itself [57].

There is also the possibility that the choice of delivery system may affect the ultimate sub-cellular distribution of the carotenoids with obvious potential for knock-on effects on biological activity. Normal sub-cellular distributions of different carotenoids are still not very well defined. Analysis of blood lymphocytes from cats, dogs, calves and pigs fed or injected with β-carotene demonstrated uptake into mitochondria, nuclei and microsomes, with mitochondria tending to contain the highest concentrations [62–65]. The description of the sub-cellular distribution of carotenoids following in vitro supplementation of cell lines is almost completely lacking. Further research in this area is required for the assessment of the value and reliability of the different delivery vehicles.

7. Application of functional genomic research

To date the scientific literature contains very few reports of the use of genomic technologies, such as microarrays and proteomics, to define the full range of the biological effects of carotenoids and unravel their precise mechanisms of action [47]. This contrasts with vitamin A, which as a therapeutic and widely used agent for promoting cellular differentiation, has been studied extensively and the signalling mechanisms exquisitely defined [66]. In 2002 Balmer and Blomhoff produced a systematic review of the literature (1191 papers) on the effects of retinoic acid on gene expression [67]. They identified 532 genes and categorised each of them according to the degree to which a hypothesis of direct versus indirect regulation by retinoic acid is supported. This massive undertaking provides an excellent basis for further studies of gene regulation by retinoic acid but it has now been superseded by a wealth of new data generated from microarray and proteomic studies [68–80]. The effectiveness and reliability of these new approaches are demonstrated by instances in which they have identified genes already well known to be regulated by retinoids (e.g. transglutaminase 2 and N-myc) [67,76,78]. In many other cases, the functional genomic approaches are generating important data that either augment previously available information describing candidate retinoid-regulated genes (e.g. EGFR, collagenases 1A1 and 1A2, chicken ovalbumin upstream promoter transcription factor 1, insulin-like growth factor binding protein 6, RXRγ and cyclin D3) or identify entirely new candidates genes (e.g. secretory protein in upper respiratory tract (spurt), galactokinase, placental bone morphogenic protein, polyamine oxidase isoform 1, and E74-like factor 3) [70,71,73,77,78]. The volume of new data demonstrates both the enormous potential of these tools for hastening discovery and the

complexity of compiling and interpreting the information they produce.

8. Conclusions

The full range of biological effects of carotenoids is being pieced together bit by bit. With the exploitation of new technologies the pace of progress should increase significantly. However, carefully considered strategies are required to make sure that the data generated in this way are of maximum value and can be compiled to provide a complete and accurate picture of the important biological effects of carotenoids and how they affect human health.

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